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Quantification of extracellular UDP-galactose

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Abstract

The human P2Y₁₄ receptor is potently activated by UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), UDP-*N*-acetylglucosamine (UDP-GlcNAc), and UDP-glucuronic acid. Recently, cellular release of UDP-Glc and UDP-GlcNAc has been reported, but whether additional UDP-sugars are endogenous agonists for the P2Y₁₄ receptor remains poorly defined. In the present study, we describe an assay for the quantification of UDP-Gal with sub-nanomolar sensitivity. This assay is based on the enzymatic conversion of UDP-Gal to UDP, using 1–4-β-galactosyltransferase. UDP is subsequently phosphorylated by nucleoside diphosphokinase in the presence of [γ ³²P]ATP and the formation of [γ ³²P]UTP is monitored by high performance liquid chromatography. The overall conversion of UDP-Gal to [γ ³²P]UTP was linear between 0.5 and 30 nM UDP-Gal. Extracellular UDP-Gal was detected on resting cultures of various cell types, and increased release of UDP-Gal was observed in 1321N1 human astrocytoma cells stimulated with the protease-activated receptor agonist thrombin. Occurrence of regulated release of UDP-Gal suggests that, in addition to its role in glycosylation reactions, UDP-Gal is an important extracellular signaling molecule.

Keywords

UDP-galactose; P2Y₁₄ receptor; nucleotide release; galactosyltransferase

INTRODUCTORY STATEMENT

Nucleotides and nucleotide-sugars are released as extracellular signaling molecules in most tissues, effecting a broad range of physiologically important responses via activation of P2X and P2Y purinergic receptors [1;2]. P2X receptors, comprising seven species (P2X₁–P2X₇), are ATP-gated ion channels. P2Y receptors belong to the superfamily of G protein-coupled receptors. At least eight P2Y receptor species have been identified in humans, seven of which (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, and P2Y₁₃) are activated by adenine and/or uridine nucleoside di- and tri-phosphates [2]. The P2Y₁₄ receptor was identified as the eighth legitimate member of the P2Y family [3;4]. Unlike other P2 receptors, the human P2Y₁₄ receptor is not activated by di- or tri-phosphonucleotides. Instead, the P2Y₁₄ receptor is activated with relatively similar potencies by UDP-glucose (UDP-Glc)¹, UDP-galactose (UDP-Gal), UDP-*N*-acetylglucosamine (UDP-GlcNAc), and UDP-glucuronic acid [3;5].

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¹The abbreviations used are: UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose; 4βGT, 1,4-β-galactosyltransferase; NDPK, nucleoside diphosphokinase; HPLC, high performance liquid chromatography.

P2Y₁₄ receptor transcripts are expressed in a broad range of human tissues, including brain, stomach, intestine, adipose, lung, spleen, and heart, and also in specialized cells such as circulating neutrophils and lymphocytes [3;6–8]. UDP-sugar-promoted signaling has been reported in astrocytes and microglial cells [9;10], lung epithelial cells [11], bone marrow hematopoietic stem cells [12], and multiple types of peripheral immune cells, including neutrophils, lymphocytes, and dendritic cells [5;7;8;13;14]. These observations suggest that UDP-sugars, high-energy donor substrates in biosynthetic reactions, are released from cells in a regulated fashion to play autocrine/paracrine signaling roles.

Recently, we demonstrated that UDP-Glc and UDP-GlcNAc are released from cells under various physiological conditions [15–19], but whether other UDP-sugars are endogenous agonist(s) for the P2Y₁₄ receptor remains to be elucidated. Several high performance liquid chromatography (HPLC) systems have been developed to resolve UDP-sugars from each other and from other nucleotides. However, a major problem in assessing nucleotides and nucleotide-sugars in extracellular solutions is that experimental conditions often require substantial dilution of the bathing medium, resulting in nucleotide concentrations that are below threshold values for standard HPLC-coupled detectors, such as UV ($\lambda = 260$ nm) or conductimetric detectors. We have recently circumvented this limitation for the measurement of UDP-Glc and UDP-GlcNAc by monitoring the UDP-Glc- and the UDP-GlcNAc-dependent conversion of [³²P]PPi to [³²P]UTP, using UDP-Glc pyrophosphorylase and UDP-GlcNAc pyrophosphorylase (AGX2), respectively [15–19]. In the present study, we describe an enzymatic assay that quantifies UDP-Gal with sub-nanomolar sensitivity. Using this assay, we investigated the presence of UDP-Gal in extracellular solutions on resting cells as well as in cells exposed to physiologically relevant stimuli.

MATERIALS AND METHODS

Reagents

UDP-Gal, α -lactalbumin, and nucleoside diphosphokinase (NDPK, EC 2.7.4.6) from Bakers yeast were purchased from Sigma. UDP-[³H]Gal (18 Ci/mmol) [³H]glucose (22 Ci/mmol), [D-glucose-1-¹⁴C]lactose (57 mCi/mmol), and [γ -³²P]ATP (3000 Ci/mmol) were obtained from Amersham Biosciences. Bovine recombinant 1,4- β -galactosyltransferase (4 β GT, E.C.2.4.1.2) was purchased from Calbiochem. All other reagents were of the highest purity available, as previously described [17;19].

Cell cultures and incubations

Polarized cultures of well-differentiated primary human bronchial epithelial (HBE) cells and Madin-Darby canine kidney (MDCK) cells were grown on 12-mm Transwell supports (Costar), as previously described [15;19]. A549 lung epithelial, 1321N1 human astrocytoma, and MCF-7 mammary carcinoma cells were grown on 24-well plastic plates (1.1 cm² per well). Cultures were rinsed three times with Dulbecco's modified essential medium (DMEM) containing 4.5 g/L glucose DMEM-H and allow to rest in 300 μ l (DMEM-H) for 1 h at 37°C in a humidified incubator supplemented with 5% CO₂ (HBE and MDCK cells were incubated in 300 μ l mucosal and 500 μ l basolateral DMEM-H). After the indicated incubation, 250 μ l medium (mucosal medium in the case of HBE and MDCK cells) was sampled and rapidly centrifuged to remove potentially detached cells. Supernatants were heated at 95°C for 2 min, and either used immediately or stored at –20°C. For intracellular measurements, cultures were lysed with 5% trichloroacetic acid, which was subsequently extracted with ethyl ether, as described [15;19].

Galactosyltransferase reactions

The conversion of UDP-Gal to lactose was assessed in 100 μ l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered DMEM-H (pH 7.4). Lactalbumin (0.2 mg/ml) was included in the lactose synthetase reaction, as indicated. The conversion of UDP-Gal to *N*-acetyllactosamine was assessed in 100 μ l glucose-free HEPES-buffered DMEM (pH 7.4) supplemented with the indicated amount of *N*-acetylglucosamine. Lactalbumin was omitted from these incubations. All 4 β GT-catalyzed reactions were performed in the presence of 5 mM MnCl₂ and the indicated amount of 4 β GT (typically, 0.002 U/ml) and substrates. Except as indicated otherwise, incubations were initiated by the addition of 4 β GT and they were allowed for 30 min at 30°C. Incubations were terminated by heating the samples for 2 min at 95°C. Samples were transferred to ice and either analyzed immediately or stored at -20°C. Species were separated by HPLC.

UDP-sugar hydrolysis

1321N1 cells were incubated in the presence of 0.1 μ Ci UDP-[³H]Gal or UDP-[³H]Glc. Samples were collected at the indicated times and heated for 2 min at 95°C to inactivate enzymatic activities potentially released from cells. Radioactive species were analyzed by HPLC.

NDPK-catalyzed phosphorylation of UDP

Unless indicated otherwise, incubations (5 min at 30 °C) were performed in 100 μ l DMEM-H containing 0.1 U/ml NDPK and 0.1 μ C [³²P]ATP (~200,000 cpm, 100 nM) and the indicated amount of UDP. The conversion of [³²P]ATP to [³²P]UTP was monitored by HPLC. To assess UDP generated from UDP-Gal released from cells, the NDPK-catalyzed reaction was preceded by a 30 min incubation of samples with or without 0.002 U/ml 4 β GT, as described above. The [³²P]UTP values obtained in the absence of 4 β GT were used to calculate UDP levels, and were subtracted from those obtained in the presence of 4 β GT to calculate UDP-Gal concentrations.

HPLC analysis

Species were separated by HPLC (Shimadzu), using the columns and solvents indicated below. The HPLC apparatus was equipped with a Radiometer Flow-One[®] Beta detector (Packard) and a SPD-10A UV detector (Shimadzu), and radioactivity and absorbance at $\lambda = 260$ nm were monitored on-line as described previously [19]. Briefly, the area of radioactive peaks was quantified using the Flo-One for Windows Radio-HPLC application software (Packard). [³²P]- and [³H]-radioactivity were measured by Cerenkov and liquid scintillography, respectively. [³²P]-peaks displaying >750 cpm (background [³²P]-background <250 cpm) and [³H]-species displaying >500 cpm ([³H]-background < 150 cpm) were analyzed (signal-to-noise-ratio >3). UDP-[³H]Gal was separated from [³H]lactose or *N*-[³H]acetyllactosamine (4 β GT reaction) or from [³H]galactose-1P and [³H]galactose (UDP-sugar hydrolysis) using a Nova-Pack C18 column (Waters) with an ion pairing mobile phase (1 ml/min) consisting of 8 mM tetrabutylammonium hydrogen sulfate and (TBEAHS) and 17 mM H₂KPO₄ (pH 5.3) in 10% methanol for the initial 5 min, and 8 mM TBEAHS (pH 5.3) and 100 mM H₂KPO₄ in 10% methanol for additional 10 min. The elution times of [³H]galactose, [³H]galactose-1P, [³H]lactose, *N*-[³H]acetyllactosamine, and UDP-[³H]Gal were 1.9, 2.5, 3.2, 3.6, and, 8.8 min, respectively. Using this HPLC system, UDP-[³H]Glc, [³H]glucose, and [³H]glucose-1P eluted at 9.2, 1.8, and 2.5, respectively. *N*-[¹⁴C]acetylglucosamine and *N*-[¹⁴C]acetyllactosamine were resolved from each other using a Kromasil-NH₂ column (Alltech) and 75% (v/v) acetonitrile as the mobile phase (1 ml/min). The elution times were 5.8 min for *N*-[³H]acetylglucosamine and 8.4 min for *N*-[³H]acetyllactosamine. [³²P]ATP and [³²P]UTP were resolved via a Hamilton PRP-X100 anion exchange column with a mobile phase (1 ml/min) consisting of 150 mM M NH₄HCO₃ (pH 8.5) in 30% methanol. UTP and ATP eluted at 8.4 and 13.5 min, respectively.

ATP and UDP-Glc measurements

The mass of ATP and UDP-Glc were assessed using the luciferin-luciferase assay and the UDP-Glc pyrophosphorylase reaction, respectively, as previously described [18].

Data analysis

The Sigma Plot 10.0 software was used for data analysis. The data displayed in Figures 3C and D were fitted according to a first-order regression line ($y = b + ax$; where y represents the % UTP formation, x is the substrate concentration, b is the intercept of $y=0$ on the y -axis, and a is the slope of the curve). The b , a , and regression coefficient squared (r^2) values are indicated in the legend of the Figure. The half-life ($t_{0.5}$) values for UDP-sugar decay (Figure 4B) were calculated as $t_{0.5} = 0.693/k$ (k is the first-order rate constant), as previously described [20]. Statistical significance (by Student's t test) was defined as $p < 0.01$.

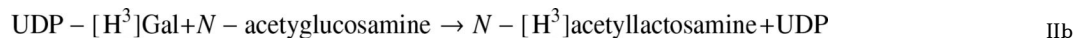
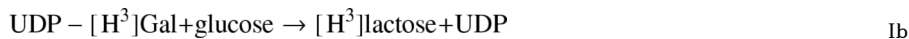
RESULTS

Synthesis of lactose and *N*-acetyllactosamine using galactosyltransferase

1,4- β -Galactosyltransferase (4 β GT) from bovine milk, also known as lactose synthetase, transfers galactose from UDP-Gal to lactose, using glucose as acceptor substrate in the presence of α -lactalbumin (equation Ia). In the absence of α -lactalbumin, *N*-acetylglucosamine is the preferred acceptor substrate of 4 β GT and lactosamine is a product of this reaction [21], as described in equation IIa.



While a number of protocols take advantage of the above reactions to assess 4 β GT activity in tissues and subcellular compartments, our goal was to use purified 4 β GT to quantify UDP-Gal in extracellular solutions. Based on previous measurements of extracellular nucleotides and nucleotide-sugars [19;22], UDP-Gal concentration in bulk extracellular solutions bathing cell cultures likely would be within the low nanomolar range, below the range of sensitivity of standard UV detectors. Therefore, we examined the possibility of using radiolabeled substrates to develop a 4 β GT-based assay that would measure the UDP-Gal-dependent formation of radiolabeled lactose or *N*-acetyllactosamine, with high sensitivity. For an initial evaluation of the efficiency of 4 β GT to catalyze the conversion of nanomolar amounts of UDP-Gal to either lactose or *N*-acetyllactosamine, UDP-[^3H]Gal was used as a radiotracer, and the formation of [^3H]-labeled products (Ib and IIb) was monitored by HPLC.



Reaction Ib was assessed in a standard glucose-containing tissue culture medium (DMEM-H) supplemented with 0.2 mg/ml α -lactalbumin and 5 mM MnCl_2 . A 60 min incubation of 0.002 U/ml 4 β GT with 0.1 μCi (100 nM) UDP-[^3H]Gal resulted in full conversion of UDP-[^3H]Gal

to [³H]lactose (Fig. 1A). The enzyme concentration-effect relationship for the lactose formation reaction is shown in Figure 1B and the time-course is illustrated in Figure 1C. As previously established for this reaction [21], conversion of UDP-[³H]Gal to [³H]lactose was negligible in the absence of α -lactalbumin (Fig. 1D).

The 4 GT-catalyzed reaction was highly efficient at all UDP-Gal concentrations tested within the 1–1000 nM range (Fig. 2A). In contrast, and consistent with the high selectivity that 4 GT exhibits towards UDP-Gal over other UDP-sugars [21;23], UDP-[³H]Glc (1–1000 nM) was recovered unchanged after a 30 min incubation with 4 GT (Fig. 2A). Thus, the lactose synthetase reaction described above quantitatively transfers galactose from UDP-Gal to lactose. However, the presence of millimolar amounts of glucose in most tissue culture media and biological samples posed a limitation for assessing UDP-Gal mass with high sensitivity, using radiolabeled glucose as radiotracer. Furthermore, using conditions (enzyme concentration, incubation time, and temperature) similar to those used above for lactose synthesis, efficient conversion of UDP-[³H]Gal (100 nM) to [³H]*N*-acetyllactosamine was achieved only at $\geq 100 \mu\text{M}$ *N*-acetylglucosamine (Fig. 2B). Thus, practical reasons prevented us from using high specific activity radiolabeled glucose or *N*-acetylglucosamine as a probe for the UDP-Gal-dependent formation of lactose or *N*-acetyllactosamine.

NDPK-catalyzed phosphorylation of UDP

To overcome the limitations associated with the use of radiolabeled acceptor substrate in the 4 β GT reaction, we investigated the possibility of measuring the formation of UDP (rather than lactose or lactosamine) as an alternative assay for UDP-Gal mass quantification. We have previously used NDPK to synthesize UTP γ S in the presence of UDP and ATP γ S [24]. Based on this and other observations [20], we recently envisioned an assay that uses NDPK to quantify the UDP-dependent conversion of [γ -³²P]ATP to [γ -³²P]UTP [25]. Therefore, we reasoned that combining the 4 β GT-catalyzed reaction (Ia) with the NDPK-based phosphorylation of UDP (reaction IIIa) would provide a reliable tool for the high sensitive measurement of UDP-Gal.



To optimize the NDPK-based assay, the enzyme concentration dependence and the time course of the reaction were assessed, and a 10-fold excess of ATP mass, relative to UDP, was added to drive the reaction towards [γ -³²P]UTP synthesis. Figure 3A indicates that, in the presence of 100 nM [γ -³²P]ATP, 10 nM UDP was nearly completely converted to [γ -³²P]UTP, using 0.1 U/ml NDPK (5 min). Under these enzyme and substrate concentrations, the reaction reached steady state (8–9% conversion of [γ -³²P]ATP to [γ -³²P]UTP) within the first min (Fig. 3B). A calibration curve for UDP (using 0.1 U/ml NDPK, 100 nM [γ -³²P]ATP, 5 min at 30°C) is illustrated in Figure 3C. As little as 0.5 nM UDP could be detected with this assay (i.e., with a signal-to-noise ratio >3), and the reaction was linear to up to 30 nM UDP (Fig. 3C and data not shown). Linearity could be expanded to 100 nM UDP by increasing the concentration of [γ -³²P]ATP to 300 nM (not shown).

Lastly, the suitability of combining 4 β GT and NDPK for the quantification of UDP-Gal was examined. Samples containing various concentrations of UDP-Gal in DMEM-H were incubated for 30 min in the absence or presence of 0.002 U/ml 4 β GT, and 0.1 U/ml NDPK and 100 nM [γ -³²P]ATP were subsequently added for an additional 5 min. As predicted, using UDP-

Gal as starting substrate, conversion of 100 nM [$\gamma^{32}\text{P}$]ATP to [^{32}P]UTP by NDPK was absolutely dependent on the presence of 4 β GT (Fig. 3D). The overall reaction displayed a sensitivity limit of 0.5 nM UDP-Gal and linearity was observed to up 30 nM UDP-Gal. Linearity could be expanded to 100 nM UDP-Gal by increasing the concentration of [$\gamma^{32}\text{P}$]ATP to 300 nM.

Cellular release of UDP-Gal

Having optimized conditions for the measurement of nanomolar amounts of UDP-Gal, we asked whether this UDP-sugar could be detected in the extracellular medium of culture cell models. Primary cultures of human bronchial epithelial (HBE) cells and various cell lines were rinsed and incubated undisturbed for 1 h in DMEM-H (0.3 ml/cm²). The medium was collected and samples were subsequently incubated for 30 min either with or without 4 β GT, followed by a 5 min incubation with NDPK and [$\gamma^{32}\text{P}$]ATP, as described above. UDP-Gal concentrations were calculated as the difference in [^{32}P]UTP formation observed between samples pre-incubated with or without 4 β GT. For comparison, UDP-Glc and ATP also were measured in these samples, using protocols previously described [19]. Extracellular UDP-Gal was present in detectable amounts under resting conditions on well-differentiated primary HBE cells and various cell lines, but was not detected (i.e., <0.5 nM) on resting human astrocytoma 1321N1 cells (Table 1). Differences in the mechanisms of release and extracellular metabolism likely accounted for the range of nucleotide levels observed within a given culture and among cell types.

An important question regarding the extracellular accumulation of UDP-Gal is whether this nucleotide-sugar is released from cells in response to physiological stimuli. Thrombin, a serine protease, promotes release of ATP and UDP-Glc from human astrocytoma cells, via activation of protease-activated receptor-1 [17;26;27]. Therefore, we took advantage of this cell model to ask to what extent thrombin-elicited UDP-Glc release is accompanied by the release of UDP-Gal. Incubation of 1321N1 cells with thrombin (20 nM, 5 min) resulted in an enhanced accumulation of extracellular UDP-Gal, i.e., from <0.5 nM to 2.3 ± 0.8 nM (Fig. 4A). The amount of UDP-Gal release detected in response to thrombin stimulation was 690 ± 242 fmol/well ($\sim 7 \times 10^5$ cells), which represented approximately 0.46% of the total cellular content of this UDP-sugar (140 pmol/well). For comparison, the increase in UDP-Glc mass in thrombin-stimulated cells represented 1090 ± 165 fmoles, a 0.36% of the cellular UDP-Glc content (305 pmol/well). Thus, relative to their intracellular levels, UDP-Gal release from thrombin-stimulated 1321N1 cells was similar (although not identical) to UDP-Glc release, suggesting a mechanism of release that reflects the relative cellular abundance of these species.

It is worth noting, however, that our measurements may have considerably underestimated the actual mass of UDP-sugar released from thrombin-stimulated cells, due to an ecto-nucleotide pyrophosphorylase (e-NPP) activity expressed on these cells [Fig. 4B and [20; 28]]. Indeed, changes in extracellular levels of UDP-Glc (Fig. 4A) and ATP (1360 ± 220 pmol/well) in response to thrombin stimulation were considerably less robust than those previously assessed in the presence of the e-NPP inhibitor β , γ -methylene ATP [17;28]. Extracellular UDP levels increased not significantly after thrombin addition (control, 2.4 ± 0.6 nM; thrombin, 3.1 ± 1.2 nM). However, we have not included β , γ -methylene ATP or other ecto-nucleotidase inhibitors in our incubations due to interferences with the NDPK-based reaction used in the UDP-Gal assay (not shown). Importantly, differences in extracellular UDP-Gal vs. UDP-Glc levels unlikely reflected differences in metabolism rates between these species, since [^3H]UDP-Gal and [^3H]UDP-Glc were metabolized on 1321N1 cells with nearly identical half-life values (8.7 and 9.9 min, respectively, Figure 4B).

DISCUSSION

The enzymatic assay for UDP-Gal mass described here provides a highly sensitive and selective approach for the quantification of this UDP-sugar in diluted biological samples. Early works by Hull and Montgomery detected UDP-Gal in cell lysates with a sensitivity of 50 pmoles, using an ion exchange HPLC method coupled with conductimetric detection [29]. Using a UV/absorbance ($\lambda = 260$ nm) detector coupled to an ion exchange HPLC column, Tomiya et al. separated and quantified various nucleotide-sugars, including UDP-Gal, with a detection limit of approximately 1 pmol [30]. However, Kochanowski et al. illustrated that the lowest UDP-Gal concentration in cell extracts capable of giving a reliable signal-to-noise ratio is 15 pmoles [31]. The sensitivities of these previously published methods are not good enough to quantify sub-nanomolar concentrations of UDP-Gal. The assay described in the current study uses 4 β GT to selectively convert UDP-Gal to UDP, and UDP is subsequently phosphorylated by NDPK using [γ^{32} P]ATP as a radiolabeled substrate. The sensitivity of this assay (50 fmol/injection or 0.5 nM in 100 μ l) is markedly greater than those previously reported. Importantly, applying the protocol described here, we quantified for the first time UDP-Gal in extracellular solutions and demonstrated the occurrence of cellular release of UDP-Gal from astrocyte-like cells in response to a physiologically relevant stimulus. Demonstration of regulated release of UDP-Gal expands previous observations with UDP-Glc and UDP-GlcNAc [17;19], providing further support for the physiological significance of the P2Y₁₄ receptor.

Earlier studies in glycobiology have indirectly suggested that release of cellular nucleotide-sugars might occur. For example, extracellular glycosyltransferase activity [32;33] was detected and extracellular glycosylation was speculated to regulate intercellular adhesion. This idea has not been unambiguously supported by other studies [reviewed in [34]]. More recently, however, Stosell and co-workers [35] illustrated that platelet surface-associated β -1,4-galactosyltransferase produced efficient galactosylation on exposed β -GlcNAc residues when UDP-Gal was added to the platelet suspension. This observation, together with our demonstration that UDP-Glc, UDP-GlcNAc, and now UDP-Gal are released from cells in a regulated manner, reopens the question of whether extracellular UDP-sugars serve extracellular roles in addition to actions as activators of G protein-coupled receptors.

We have not addressed the issue of the mechanism of UDP-Gal release, but an attractive hypothesis is that the lumen of the secretory pathway is an important source of extracellular UDP-sugars. UDP-sugars are synthesized in the cytosol and transported to the lumen of the endoplasmic reticulum (ER) and/or Golgi apparatus to serve as sugar donors for glycosyltransferase-catalyzed reactions. ER/Golgi-resident nucleotide sugar transporters translocate cytosolic UDP-sugars to these organelles, using luminal UMP as antiporter substrate [36]. UDP-sugars imported to the ER/Golgi reach concentrations up to 20-fold higher than cytosolic levels [36]. Since ER/Golgi nucleotide-sugars are not transported back to the cytosol, potentially they can be delivered as cargo molecules and released from cells, e.g., during export/secretion of glycoconjugates. Hence, vesicular UDP-sugar release should be highly dependent on the ER/Golgi expression of the cognate transporter. Indeed, we have recently demonstrated that ER/Golgi UDP-GlcNAc transporters contribute to the release of UDP-GlcNAc from epithelial cells and yeast [19].

In conclusion, we describe a novel assay that allows the quantification of UDP-Gal with sub-nanomolar sensitivity. We have demonstrated that UDP-Gal, a potent agonist at the cell surface P2Y₁₄ receptor, is present in the conditioned medium of various cell models, including physiologically relevant primary cultures of human bronchial epithelial cells. We also illustrated that enhanced release of UDP-Gal occurs under physiological/pathophysiological conditions, such as in thrombin-stimulated astrocytes. Given the likelihood that release of the

activating P2Y₁₄ receptor ligand may differ according to cell type, UDP-Gal predictably is an important autocrine/paracrine regulator of P2Y₁₄ receptor activity.

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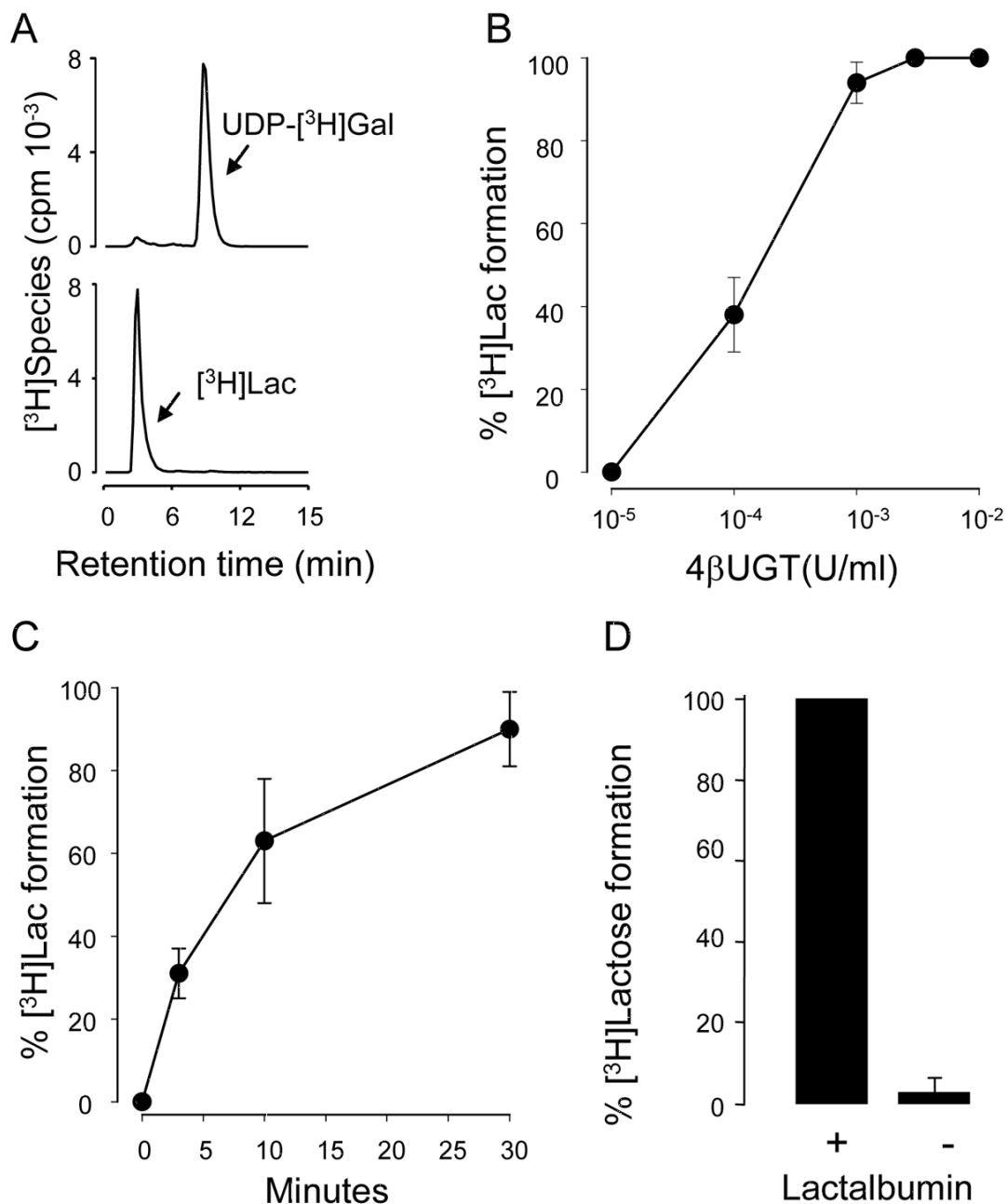


Figure 1. Enzymatic conversion of UDP-[³H]Gal to [³H]lactose

A, HPLC tracings illustrating the conversion of UDP-[³H]Gal to [³H]lactose ([³H]Lac) in the presence (bottom) or in the absence (top) of 4βGT (0.002 U/ml, 60 min, 30°C). B, incubations were for 60 min at 30°C in the presence of the indicated amount of 4βGT. C, the time-course of the reaction was examined using 0.002 U/ml 4βGT. D, the conversion of UDP-[³H]Gal to [³H]lactose was assessed in the absence (-) or presence (+) of 0.2 mg/ml α-lactalbumin. All reactions were performed in 100 μl DMEM-H containing 20 mM glucose, 100 nM (0.1 μCi) [³H]UDP-Gal, 5 mM MnCl₂, and (except as indicated in D) 0.2 mg/ml α-lactalbumin. Radioactive species were separated by HPLC. The data represent the mean ± SD from at least three independent experiments performed in duplicates.

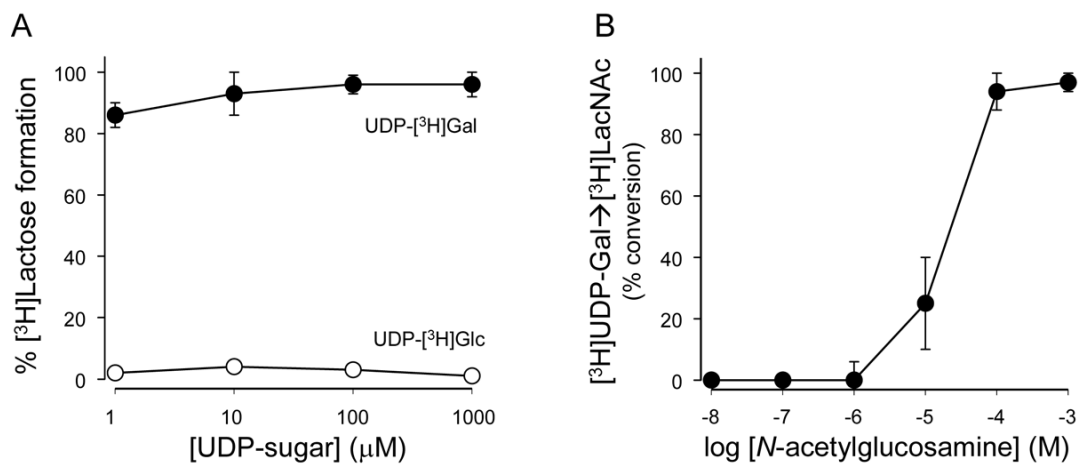


Figure 2. 4 β GT-catalized formation of lactose or N-acetyllactosamine

A, UDP-Gal but not UDP-Glc is a substrate of 4 β GT. Incubations were as in Figure 1A, in the presence of the indicated concentration of UDP-[^3H]Gal or UDP-[^3H]Glc. B, effect of *N*-acetylglucosamine concentration on the conversion of 100 nM (0.1 μCi) UDP-[^3H]Gal to [^3H] *N*-acetyllactosamine. Radioactive species were separated by HPLC. The data represent the mean \pm SD, $n \geq 3$.

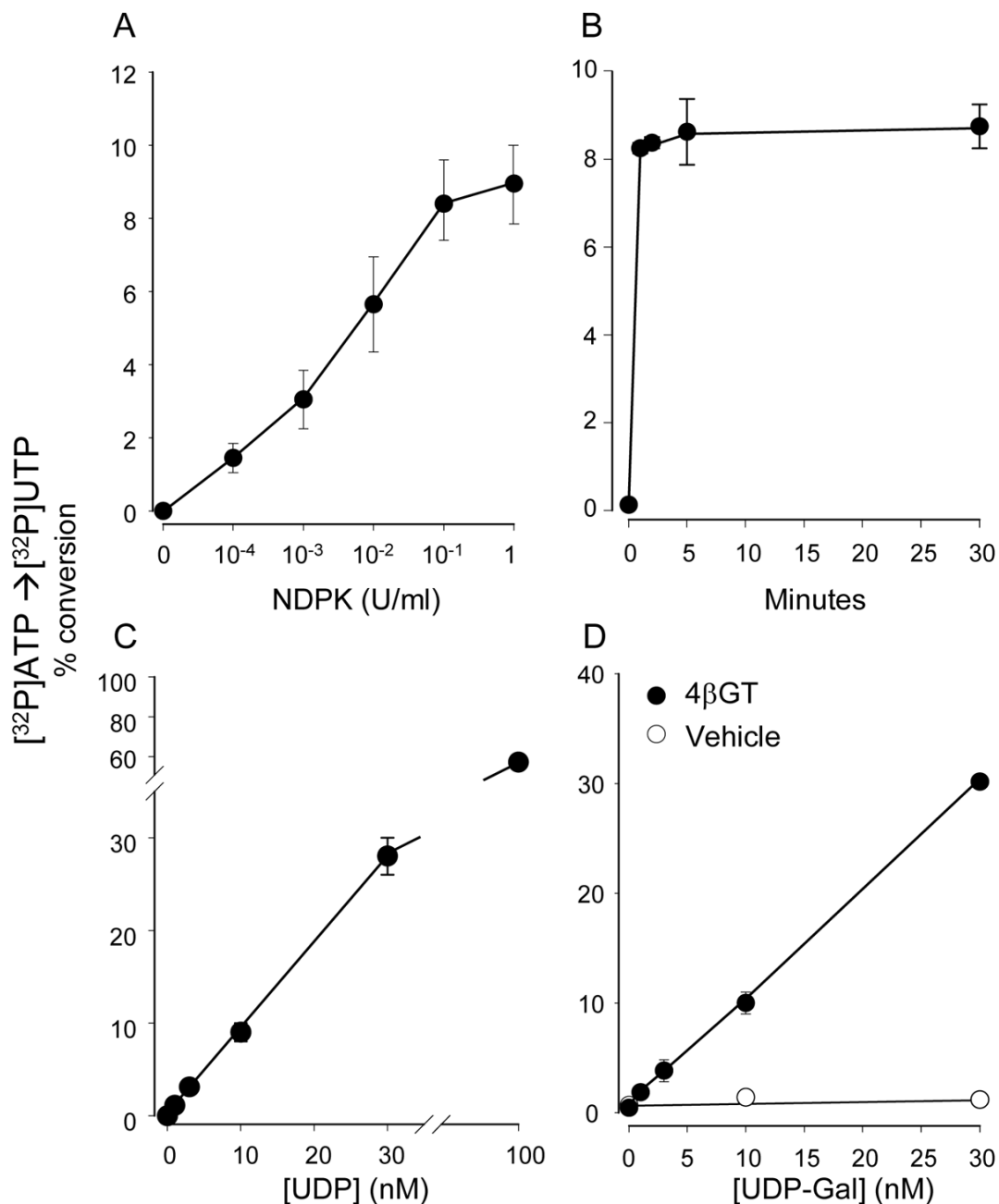


Figure 3. Assessing UDP-Gal via UDP phosphorylation

The conversion of $[\gamma^{32}\text{P}]\text{ATP}$ to $[\gamma^{32}\text{P}]\text{UTP}$ was assessed by HPLC, as detailed in Methods. A, UDP (10 nM) was incubated in the presence of 100 nM (0.2 μCi) $[\gamma^{32}\text{P}]\text{ATP}$ and the indicated amount of NDPK. B, time course of the NDPK (0.1 U/ml)-catalyzed phosphorylation of 10 nM UDP in the presence of 100 nM (0.2 μCi) $[\gamma^{32}\text{P}]\text{ATP}$. C, the UDP calibration curve was performed in the presence of 0.1 U/ml NDPK and 100 nM (0.2 μCi) $[\gamma^{32}\text{P}]\text{ATP}$ (5 min, 30°C). The reaction was linear between 0–30 nM UDP ($r^2 = 0.9996$), displaying a slope a value of 0.93784 and an intercept b value of 0.07. D, UDP-Gal (at the indicated concentration) was incubated for 30 min in the absence or presence of 0.002 U/ml 4 β GT. At the end of this period, NDPK and $[\gamma^{32}\text{P}]\text{ATP}$ were added for 5 min, as detailed in C. The reaction was linear between

0–30 nM UDP-Gal ($a = 1.016$, $b = 0$, and $r^2 = 0.9998$). The data represent the mean \pm SD, $n \geq 4$. All incubations were performed in 100 μ L DMEM-H supplemented with MnCl_2 and α -lactalbumin, as indicated in Figure 1. Background radioactivity (i.e., observed in the absence of UDP-Gal and UDP) was subtracted from all data points to allow regression lines fitting to origin.

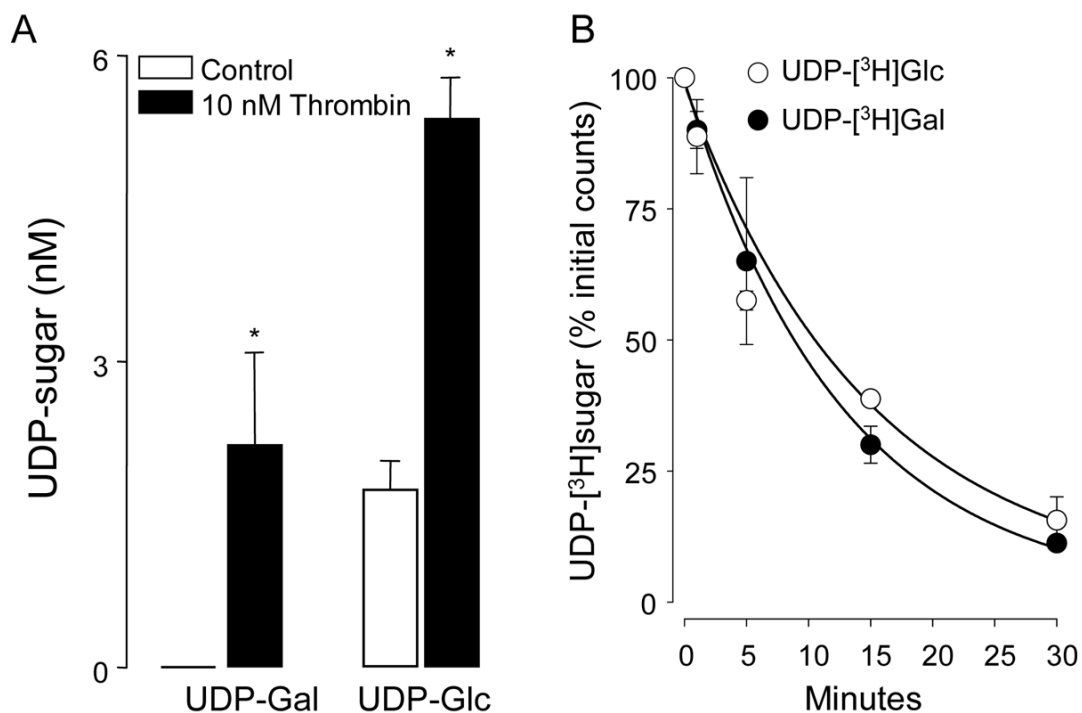


Figure 4. Enhanced UDP-Gal and UDP-Glc release from thrombin-stimulated 1321N1 human astrocytoma cells

A, cells were rinsed and pre-incubated in 300 μ l DMEM-H for 1 h, and thrombin (20 nM) was subsequently added for 5 min. UDP-Gal and UDP-Glc present in the extracellular medium were quantified, as described in Methods. The results represent the main value (\pm SD) from two independent experiments performed in triplicate; (*) indicates significant differences relative to control ($p < 0.01$, t -test). B, the stability of extracellular UDP-Gal and UDP-Glc on 1321N1 cells was assessed in cultures spiked with 0.5 μ Ci of the indicated radiotracer. Samples were collected at the indicated times and the resulting [³H]species were analyzed by HPLC, as described in Methods (mean \pm SD, $n = 4$).

Table 1
Extracellular nucleotide levels on resting cells

Cells were rinsed and incubated undisturbed for 1 h in DMEM-H. The medium was collected and nucleotides measured as indicated in Methods. The data (expressed in nM) represent the mean \pm SEM from at least two independent cultures with quadruplicate samples.

Cell type	UDP-Gal	UDP	UDP-Glc	ATP
HBE	1.5 \pm 0.4	1.7 \pm 0.2	4.6 \pm 2.2	2.5 \pm 0.5
MDCK	9.1 \pm 3	8.2 \pm 2	32 \pm 4.2	1.2 \pm 0.2
1321N1	< 0.5	2.4 \pm 0.8	1.5 \pm 0.8	4.2 \pm 0.5
MCF-7	1.8 \pm 0.5	2.7 \pm 1.1	2.9 \pm 0.4	6.1 \pm 1.0
A549	0.7 \pm 0.3	3.2 \pm 0.6	2.3 \pm 1.5	11 \pm 3.3